The *Trypanosoma brucei* sphingolipid synthase, an essential enzyme and drug target

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Abstract

Sphingolipids are important components of eukaryotic membranes, particularly the plasma membrane, and are involved in a diverse array of signal transduction processes. In the Eukaryota the biosynthetic pathway for the formation of these lipid species is largely conserved. However, in contrast to mammals which produce sphingomyelin (SM), several pathogenic fungi and protozoa synthesize inositol phosphorylceramide (IPC) as the primary phosphosphingolipid. This process is catalyzed by the enzyme IPC synthase, a recognized target for anti-fungals encoded by the *AUR1* gene in yeast. Recently, functional orthologues of the AUR1p have been identified in a group of insect vector-borne pathogenic protozoa, the Kinetoplastida, which are responsible for a range of so-called neglected diseases. Of these the *Trypanosoma brucei* species are the causative agents of human African trypanosomiasis in many of the most under-developed regions of Africa. The available treatments for these diseases are limited, of decreasing efficacy, and often demonstrate severe side-effects. Against this background the *T. brucei* sphingolipid synthase, an orthologue of the yeast AUR1p, may represent a promising target for novel anti-protozoals. Our studies identify an isoform of this protein as a novel bi-functional enzyme capable of catalyzing the synthesis of both IPC and SM, both known to be present in the parasite. Furthermore, the synthase is essential for parasite growth and can be inhibited by a known anti-fungal at low nanomolar levels *in vitro.* Most notably this drug demonstrates trypanocidal activity against cultured bloodstream form parasites. Thus, the *T. brucei* sphingolipid synthase represents a valid and promising drug target.

Key words

Trypanosoma, trypanosomiasis, sphingolipid synthase, drug target

Footnote

Since the initial submission of this work to Molecular and Biochemical Parasitology a paper has been published in Molecular Microbiology identifying and characterising the same family of enzymes in *Trypanosoma brucei*:

Sutterwala S.S., Hsu F.F., Sevova E.S., Schwartz K.J., Zhang K., Key P., Turk J., Beverley S.M., Bangs J.D. Developmentally regulated sphingolipid synthesis in African trypanosomes (2008) 70:281-296.

1. Introduction

Trypanosoma brucei species are protozoan parasites of the order Kinetoplastidae and the etiological agents of both human African trypanosomiasis (HAT, sleeping sickness), and diseases of economically important animals (e.g. nagana in cattle) [1]. These diseases are endemic in much of sub-Saharan Africa, with HAT causing a burden of approximately 1.6 million disability adjusted life years (http://www.who.int/tdr/). This distribution across some of the most under developed regions of the world is coupled with a paucity of effective therapies, with those available being either too expensive (e.g. eflornithine) or exhibiting catastrophic side-effects (e.g. melarsoprol). Together with the leishmaniases (caused by the related *Leishmania* species) HAT has been described as an emerging or uncontrolled disease. Therefore, there is an urgent need for new, validated drug targets and anti-HAT compounds to combat a disease causing in excess of 50,000 deaths per annum [2].

Sphingolipids are a diverse group of amphipathic lipids that perform essential functions in eukaryotes. For example, the unmodified sphingolipid ceramide acts as a secondary signalling molecule [3] and more complex species are implicated in the formation and function of signal transduction complexes [4, 5]. The primary phosphosphingolipid species in mammalian species, including humans, is sphingomyelin (SM). SM is formed by the transfer of the phosphorylcholine head group from phospholipid phosphatidylcholine (PC) to ceramide, a reaction catalyzed by SM synthase [6]. In contrast fungi, plants and at least some protozoa produce inositol phosphorylceramide (IPC) as their primary phosphosphingolipid [7]. In these organisms IPC synthase catalyzes the transfer of phosphorylinositol from phosphatidylinositol (PI) to ceramide [8-

10]. IPC synthase has long been established and studied as a target for novel anti-fungals [11, 12]. More recently this enzyme has come under scrutiny as a potential target for anti-protozoals [13]. With the recent identification and characterization of the *Leishmania* IPC synthase (LmIPCS; [10]) it has become possible both to validate this protozoan activity as a drug target and, furthermore, begin to investigate potential inhibitors, including those known to act against the fungal IPC synthase. Four closely related orthologues of LmIPCS are apparent in the *T. brucei* database [10], and mass spectrometry of isolated fractions has revealed that whilst the predominant phosphosphingolipid in pathogenic bloodstream form parasites is SM [14]; insect stage, procyclic *T. brucei* also contain IPC [15].

Here we describe the characterization of the African trypanosome, *T. brucei,* sphingolipid synthase 4 (TbSLS4) which demonstrates itself to be a novel bi-functional enzyme with the ability to catalyze the biosynthesis of both IPC and SM, thus reflecting the sphingolipid profile of the parasite. Importantly, the IPC synthase activity of TbSLS4 is acutely sensitive to the well characterized specific fungal inhibitor aureobasidin A [11, 12] and pathogenic bloodstream form *T. brucei* are rapidly killed at sub-micromolar concentrations of this drug. Furthermore, down-regulation of TbSLS1-4 using inhibition RNA (RNAi) in bloodstream form parasites demonstrated that the enzyme activity is essential for growth thus validating it as a target for the development of new anti-HAT therapies.

2. Materials and methods

2.1. Functional identification of the *Trypanosoma brucei* sphingolipid synthase

A common, conserved AUG start codon was predicted for all 4 TbSLS isoforms by examination of the genome sequence (www.genedb.org). Subsequently, TbSLS1 (Tb09211.1030) and TbSLS4 (Tb09211.1000) were amplified with *Pfu* polymerase (Promega) from *T. brucei* strain Lister 427 genomic DNA using primer pairs (homologous sequence underlined):

TbSLS1 - CCGGAATTCATGATTAGTTACCCTTTCTTCTCCC and

CCGCTCGAGTCATACCTCGTTAGTTGATAC

TbSLS4 – CCGGAATTCATGATTAGTTACCCTTTCTTCTCCC and

CCGCTCGAGTCACACATACGCCCCACATTTAAAC;

The PCR products were subsequently cloned into the yeast expression vector pRS426 MET [16] to give pRS426 TbSLS1 and pRS426 TbSLS4. These, together with pRS426 AUR1, pRS426 human sphingomyelin synthase 1 and 2 (HsSMS1 and 2) and empty vector (pRS426), were used to transform the YPH499–HIS–GAL–AUR1 *S. cerevisiae* strain [10]. Transformants were selected on non-permissive SD medium (0.17% Bacto yeast nitrogen base, 0.5% ammonium sulphate and 2% dextrose) or permissive SGR medium (0.17% Bacto yeast nitrogen base, 0.5% ammonium sulphate, 4% galactose and 2% raffinose) containing the appropriate nutritional supplements at 30°C.

2.2. Metabolic labelling and analyses

Yeast were grown to exponential phase in SD or SGR as indicated and 2.5 OD_{600} units incubated in 1 ml of SD or SGR supplemented with 5 μM of NBD C_6-ceramide (Invitrogen) conjugated to fat-depleted bovine serum albumin (Sigma-Aldrich) for 120 minutes at 30°C. Yeast were harvested by centrifugation and washed twice with phosphate buffered saline. Chloroform/methanol (0.4 ml; 1:1 v/v) was added and cells were disintegrated with glass beads. The pellet was re-extracted with chloroform/methanol/water (10:10:3) and the lipid fraction isolated by phase separation. After drying in a rotavapor (Eppendorf Concentrator 5301) reaction products were re-suspended in 20 *μ*l of 10:10:3 and cell mass equivalents fractionated using HPTLC silica plates (Merck) and the eluent system chloroform:methanol:aqueous 0.25% KCl (55:45:10). Imaging and quantification was carried out using a FLA3000 scanner (Fujifilm) and AIDA Image Analyzer® software (version 1.3). Vero cells were labelled and processed for use as controls and standards as previously described [10].

Sphingomyelinase (*Bacillus cereus*; Sigma Aldrich) was used to identify sphingomyelin in the NBD C_6-ceramide lipids extracted from the complemented yeast as previously described [17]. Briefly, YPH499–HIS–GAL–AUR1 *S. cerevisiae* complemented with pRS426 TbSLS4 (in SD) or transformed with pRS426 HsSMS2 or empty vector (in permissive SGR, or for a limited period SD) were grown to exponential phase. After adjustment to an optical density of 0.5 OD_{600} in 5 ml of either SD or SGR, cells were labelled for 16 hours with 2 μM of NBD C_6-ceramide conjugated to fat-depleted bovine serum albumin as previously described [6]. Labelled lipid fractions from these were prepared and dried as above with 50 *μ*g of sphingomyelin (Sigma Aldrich), and

resuspended, with sonication in a water bath, in 600 μl of 20 mM Tris-HCL (pH7.4), 10 mM $MgCl_2$ and 0.05% (w/v) Triton X-100. Subsequently, samples (300 μl) were incubated with or without 2 units of *Bacillus Cereus* sphingomyelinase (Sigma Aldrich) at 37°C for 120 minutes. Equivalent lipid extracts were fractionated and analyzed as above.

2.3. *In vitro* assay of TbSLS4 activity

Microsomal membranes from exponentially growing YPH499–HIS–GAL–AUR1 pRS426 TbSLS4, pRS426 LmIPCS or pRS426 AUR1 [10] were prepared as previously described [18] and the isolated membrane fraction re-suspended in storage buffer (50 mM Tris/HCl pH 7.4, 20% (v/v) glycerol, 5 mM $MgCl_2$) with Complete® EDTA-free Protease Inhibitor Cocktail (Roche Applied Science) at a protein concentration of 10 mg/ml. The microsomal membranes were subsequently washed in 2.5% CHAPS (w/v; Sigma Aldrich; 4 °C, 60 minutes), isolated by centrifugation (150000 g, 4 °C and 90 min), re-suspended in storage buffer at 10 mg/ml and stored at −80 °C until use.

The assay mix contained 100 μM donor substrate (bovine liver PI, PC or PE, Avanti Polar Lipids), 10 μg of microsomes, 100 mM Tris HCl, 10 mM EDTA, 6 mg/ml BSA and 5 μM NBD C_6-ceramide [19]. Following incubation at 30 °C for 60 minutes the reaction was quenched by the addition of 150 μl of chloroform:methanol:water (10:10:3). After biphasic separation the organic layer was removed, processed, quantified and analyzed as above.

For inhibition experiments the reaction mix was pre-incubated for 30 minutes with appropriate quantities of aureobasidin A (Takara) before the addition of NBD C_6-ceramide.

2.4. Parasite culture

Bloodstream form *T. brucei* strains Lister 427 and its engineered variant, Single Marker Bloodstream form (SMB, T7RNAP::TETR::NEO; [20]) were maintained *in vitro* at 37°C with 5% CO_2 in HMI-9 medium supplemented with 10% FCS and, for SMB, 2.5 μg/ml G418.

2.5. Inhibition RNA (RNAi) of TbSLS

An 165 base pair sequence fragment common to all four TbSLS open reading frames was amplified from genomic DNA using *Pfu* polymerase and primer pair (homologous sequence underlined): CATAGATCTAGAGGTTCCATACACTGTG and CATAGATCTAGACGAGAGGCAACGATGC

This PCR product was cloned into the RNAi vector p2T7 [21] and, following linearization, 10 μg transfected into SMB *T. brucei* and transformants selected using 2 μg/ml phleomycin (Sigma Aldrich). Following induction with 1 μg/ml doxycycline cell growth was determined at 24 hour intervals by light microscopy using an Improved Neubauer Haemocytometer.

48 hours post-induction total RNA was isolated (RNeasy®, Qiagen) and RT-PCR was performed (SuperScript II, Invitrogen) using the primer pairs:

TbSLS: AAACTGTACCTTCTTCACCG and CGAGAGGCAACGATGC;

Tb β tubulin: GGAGCGCATCAATGTGTAC and CAGGCAGCAGGTGACGCCG

2.6. *T. brucei* susceptibility to aureobasidin A

T. brucei Lister 427 were cultured in the presence of various concentrations of aureobasidin A. Growth was analyzed at 24 hour intervals as above.

3. Results and discussion

3.1. Identification and characterization of the *T. brucei* sphingolipid synthase

Four tandem gene sequences (Tb09211.1030, Tb09.211.1020, Tb09.211.1010 and Tb09.211.1000; here annotated TbSLS1-4 to reflect their 5' to 3' order) were previously identified in the *T. brucei* genome database (www.genedb.org) as sequence orthologues of the inositol phosphorylceramide synthase (LmIPCS) from the related kinetoplastid parasite, *Leishmania* [10]. The predicted open reading frames encode 4 closely related trans-membrane proteins with more than 90% identity and 94% similarity. Most variation occurs at the carboxy-termini, a region predicted to lie on the cytosolic side of the membrane away from the active site at the Golgi lumen, with another variable domain close to and within the second predicted trans-membrane domain [10]. One of the predicted *T. brucei* sphingolipid synthase isoforms (Tb09.211.1000; TbSLS4) was the focus of this study. In addition, the isoform most distant from TbSLS4 with respect to the internal variable domain (Tb09.211.1030; TbSLS1) was also subjected to preliminary analyses.

The auxotrophic mutant *Saccharomyces cerevisiae* strain, YPH499-HIS-GAL-AUR1, has the essential *AUR1* IPC synthase gene under the control of a galactose inducible promoter. Therefore it is unable to grow in the presence of the repressor glucose, a phenotype that is rescued by the ectopic expression of LmIPCS [10]. Similarly, TbSLS4 and TbSLS1 expression complemented the YPH499-HIS-GAL-AUR1 mutant yeast line indicating that they are also functional orthologues of the yeast *AUR1* gene (figure 1). The yeast IPC synthase (AUR1) also complemented this mutant line. Significantly, neither HsSMS1 nor HsSMS2 complemented the YPH499-HIS-GAL-AUR1 yeast (data

not shown) indicating that sphingomyelin synthase activity alone is not sufficient to rescue the mutant.

To understand the function of the *T. brucei* sphingolipid synthase, the auxotrophic YPH499-HIS-GAL-AUR1 yeast cells complemented with either TbSLS4, TbSLS1 or yeast AUR1 were metabolically labelled with fluorescent NBD C_6-ceramde, a substrate for sphingolipid synthases, including those from the kinetoplastids [10]. Under the conditions described, the AUR1 complemented *S. cerevisiae* auxotrophic mutant synthesized IPC as the only labelled product. In contrast, both TbSLS4 and TbSLS1 complemented mutant yeast were shown to synthesize two major labelled lipid species. One of these co-migrated with SM, the other with IPC. The latter at levels equivalent to those produced in the AUR1 complemented yeast (figure 2A). In addition, TbSLS4 complemented yeast synthesized a third species which co-migrated with an unknown lipid (X) produced by labelled mammalian cells (Vero). As a control YPH499-HIS-GAL-AUR1 cells harbouring an empty vector (pRS426) were cultured in both permissive (SGR) and non-permissive (SD) media. Both lines grew equivalently in both media for 16 hours and for a further 8 hours after dilution to an optical density 0.3 OD_{600}, and remained viable (by plating on permissive media, data not shown). Labelling of these dividing cells with NBD C_6-ceramde in their respective media under the same conditions as above demonstrated that in non-permissive SD no labelled IPC is produced, indicating the down-regulation of AUR1p (figure 2B).

TbSLS4 was chosen for further study due to the relative predominance of the SM-like species in the labelled complemented yeast. Unlike IPC, SM is known to be present in the pathogenic bloodstream form of the parasite [14]. The labelled SM-like species (and

the unknown X) proved to be susceptible to sphingomyelinase (which breaks down SM into phosphorylcholine and ceramide) when lipid extracts were treated with this enzyme, thereby confirming its identity (figure 3A). In contrast the IPC produced was insensitive to this enzyme treatment. Extracts from the auxotrophic mutant expressing HsSMS2, which produce an equivalent quantity of labelled SM under permissive conditions (SGR), acted as a control for SMase activity.

Together these data suggest that TbSLS4 is a novel bi-functional enzyme acting as both a SM and an IPC synthase. This is consistent with previous analyses which demonstrated that the parasite harbours both SM [14] and IPC phosphosphingolipids [15].

3.2. *In vitro* analyses of TbSLS4 activity

To further investigate the function of TbSLS4, microsomal material was isolated from the TbSLS4 and AUR1 complemented yeast as described and used in an *in vitro* assay utilising the common acceptor substrate NBD C_6-ceramide and the candidate donor substrates bovine liver PI, PC and phosphatidylethanolamine (PE; a potential substrate for ethanolamine phorphorylceramide synthesis [22]). Notably, when assayed, the yeast AUR1 crude microsomal material showed significant IPC synthase turnover, but demonstrated little significant increase in this on the addition of the donor substrate PI. As expected, no detectable SM synthesis was observed with or without the addition of PC (figure 4A). In order to clearly assign enzyme function this assay was refined according to data obtained from the analysis of LmIPCS (Mina *et al.* in preparation). In brief, microsomal fractions were washed with ice-cold 2.5% 3-[3-(cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) to remove endogenous (i.e. yeast)

substrates. This facilitated analyses of the effect of adding exogenous substrate to the reaction – in this case the candidate donor substrates (PI, PC or PE) plus the known acceptor substrate NBD C_6-ceramide. Without the addition of the donor substrate the CHAPS-washed AUR1 microsomes, compared to the crude unwashed sample, demonstrated a relatively low level of IPC synthase turnover and, as expected no evidence of SM synthase function (figure 4A, Crude and Washed). Importantly, the addition of PI or PC had no discernable effect on enzyme turnover in the washed sample indicating that the yeast IPC synthase is either substrate specific (and unable to utilise bovine PI) or that the detergent treatment had disrupted the protein (figure 4A, +PI and +PC Washed). In contrast, assay of identically treated TbSLS4 microsomes showed that the addition of PI led to a large (more than 12-fold) increase in the formation of IPC (figure 4B, +PI). This demonstrated that TbSLS4 functions as an IPC synthase, an activity not attributable to background AUR1 expression.

Surprisingly given the identification of SM as a TbSLS4 product above (figure 3A), bovine PC – a potential donor substrate for a SM synthase - had no significant effect on enzyme activity (figure 4B, +PC). The addition of PE was similarly ineffectual (figure 4B, +PE). However, when PC or PE were added simultaneously as molar equivalents with PI to the assay system, the quantity of IPC produced decreased 4 and 2-fold respectively. This suggested that both PC and PE bind competitively with PI to the same region of the enzyme (figure 4B, +PI+PC and +PI+PE).

Taken together these results indicate that TbSLS4 functions as an IPC synthase but also binds PC (and PE) and can participate in the synthesis of SM. The lack of *in vitro* SM synthase activity in the presence of exogenous PC is surprising and may relate to

substrate specificity (see yeast AUR1 and bovine PI, figure 4A, Washed). One possibility is that TbSLS4, in this assay, is unable to utilize bovine liver PC. This donor substrate is a mixed natural product with predominantly C36:2 PC. In contrast, *S. cerevisiae*, where both TbSLS4 and TbSLS1 function as SM synthases, predominantly possesses C32:2 and C34:2 PC [23]. Perhaps TbSLS favours these relatively short acyl groups? However, *T. brucei* procyclic and bloodstream forms harbour significant quantities of C36, C38 and C40 PC species [14, 24, 25], indicating that any substrate selectivity is perhaps due more subtle structural differences. In support of this, although TbSLS4 can utilise bovine liver PI (predominantly C38:4) efficiently as a substrate for IPC synthesis, procyclic form parasites (known to synthesize IPC [15]) harbour only trace levels of the C38:4 PI [14].

3.3. Inhibition of TbSLS4 using a known anti-fungal agent

IPC synthase is a recognized target for anti-fungal drugs and the natural product aureobasidin A is a widely utilized and specific experimental inhibitor [26]. This drug also specifically inhibits the activity of *Leishmania* LmIPCS, a TbSLS orthologue, albeit at a concentration several orders of magnitude higher than those for the *S. cerevisiae* enzyme [10].

To establish the efficacy of aureobasidin A against the *T. brucei* enzyme the previously described *in vitro* assay was employed using CHAPS-washed TbSLS4 microsomes with NBD C_6-ceramide and bovine PI as receptor and donor substrates respectively. As a control identically prepared LmIPCS microsomes were assayed in the same manner. Given the inactivity of the yeast enzyme in this assay system it was not possible to include this as a further control. The synthesis of labelled IPC was used as a measure of

IPC synthase turnover. From this assay it was evident that the *T. brucei* enzyme is acutely sensitive to the drug (figure 5A). Following reanalysis of the linear portion of the curve (0 – 0.5 nM aureobasidin A), the turnover was calculated to be 50% inhibited (IC_{50}) by 0.42 nM aureobasidin A. TbSLS4 turnover was undetectable at a concentration of 50 nm aureobasidin A. In contrast, the IC_{50} for LmIPCS inhibition by aureobasidin A was more than 200,000 times higher, with precipitation of the drug at concentrations above 100 μM (figure 5B) preventing the determination of an absolute value.

3.4. Validation of TbSLS as a target of anti-protozoals

Using a sequence fragment common to all four TbSLS isoforms an RNAi construct was prepared in the p2T7 vector [21] and used to specifically inhibit TbSLS expression in cultured bloodstream form *T. brucei* (SMB). Non-induced TbSLS RNAi cells grew in a similar manner to control SMB parasites carrying empty vector with or without the doxycycline. In contrast, doxycycline induction of TbSLS RNAi saw the parasites cease division and led to some cell death as scored by light microscopy. RT-PCR, using β tubulin as a control, confirmed the specificity of the TbSLS mRNA inhibition (figure 6).

This genetic approach validated TbSLS as an essential enzyme for pathogenic bloodstream form parasite growth. Given the *in vitro* data shown above in which aureobasidin A was demonstrated to inhibit TbSLS4, the efficacy of this natural compound was tested against cultured bloodstream form *T. brucei* (Lister 427; figure 7). When the concentration of aureobasidin A was 1 μM cell growth was completely inhibited and the parasites were scored as dead by light microscopy after 24 hours. The EC_{50} of aureobasidin A against the parasites was estimated from these data as being

below 250 nM. However, it is the trypanocidal activity of this compound at higher concentrations that is of most significance.

3.5. Summary

Previous studies using pharmacological and genetic inhibition of the first step in sphingolipid biosynthesis, catalysed by serine palmitoyltransferase, showed that this pathway is essential for the viability of both bloodstream and procyclic forms of *T. brucei* [17, 27]. This is in contrast to the related protozoan parasite *Leishmania major* where this enzyme, though essential for sphingolipid biosynthesis, is non-essential for both viability and pathogenesis [28, 29]. These studies indicate that sphingolipid biosynthesis could be a viable drug target in the African trypanosomes.

The sphingolipid biosynthetic pathway is largely conserved across the Eukaryota. However, whilst animal cells synthesize the phosphosphingolipid SM, yeast and plants, plus at least some protozoa, produce IPC [7]. The IPC synthase of pathogenic fungi has long been validated and studied as a drug target [11], and the recent identification of a functional orthologue in the protozoan Kinetoplastids, the causative agents of several so-called neglected diseases, has led to its consideration as a target for anti-protozoal agents [10]. In this study we confirm that 2 of the 4 closely related *T. brucei* orthologues (TbSLS4 and 1) of the *Leishmania* IPC synthase (LmIPCS) are also functional orthologues of the *S. cerevisiae* enzyme encoded by *AUR1*. However surprisingly, unlike the *Leishmania* enzyme (and AUR1) TbSLS4 and 1 are able to catalyze the synthesis of both IPC and SM, which reflects the known sphingolipid content of *T. brucei* cells [15]. In an *in vitro* assay system utilizing TbSLS4 complemented *AUR1* mutant yeast microsomes, it was demonstrated that the *T. brucei* enzyme was able to function as an IPC synthase. Although PC (and PE) was demonstrated to be a competitive binder with respect to PI, SM synthase activity could not be constituted possibly due to

some level of specificity with respect to the donor substrate PC. Clearly, substrate specificity and the mechanism of action of TbSLS4 warrant further investigation to disentangle the detected IPC synthase function from the apparent SM synthase activity. Determination of the kinetic parameters of TbSLS4 would facilitate this, for example with respect to determining the binding constants for the apparent competitive IPC synthase inhibitors - PC and PE. In this respect it should be noted that the *T. brucei* enzyme is unlike its orthologues from the other kinetoplastid parasites where both the *Leishmania* (figure 5B) and *T. cruzi* enzymes (Casbon and Denny, unpublished) demonstrate only IPC synthase activity in the *in vitro* system employed here. It is also clearly important to fully analyse the other 3 TbSLS isoforms to determine their function.

The known yeast and fungal IPC synthase inhibitor, aureobasidin A, has previously been shown to be active against the related kinetoplast, *Leishmania* species, inhibiting growth, but not affecting viability, in culture [30]. However, it has been demonstrated that the *L. major* IPC synthase is refractory to aureobasidin A (figure 5B) and that its affect against this species in culture is non-specific [28]. A similar situation has been observed with respect to the causative agent of Chagas disease, *T. cruzi* [19]. In contrast, this study showed that aureobasidin A demonstrated a high level of efficacy against TbSLS4 turnover *in vitro*, with an IC_{50} of 0.42 nM. In light of these results demonstrating the ability of a known inhibitor to affect enzyme activity, it was important to validate TbSLS as a potential target of anti-trypanosome drugs. Simultaneous RNAi of all four closely related isoforms of TbSLS demonstrated that this enzyme is essential for growth and so represents a new, much needed anti-protozoal target. Furthermore, aureobasidin A

proved highly effective and trypanocidal against cultured bloodstream form *T. brucei*, with a sub-micromolar EC_{50}.

Together these data raise the possibility of the discovery of a new generation of lead inhibitors directed against TbSLS, ultimately leading to novel drugs for the treatment of human African trypanosomiasis.

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Figure legends

Figure 1

Transformation with pRS426 TbSLS4 and pRS426 TbSLS1, as well as pRS426 AUR1 (the *S. cerevisiae* IPC synthase), rescues the auxotrophic mutant YPH499-HIS-GAL-AUR1 allowing it to grow in the presence of glucose (SD media). YPH499-HIS-GAL-AUR1 pRS426 (empty vector) does not grow in the presence of glucose (SD), however all lines are viable in the absence of glucose and the presence of galactose (SGR).

Figure 2

A. Metabolic labelling of YPH499-HIS-GAL-AUR1 yeast IPC synthase (AUR1) complemented yeast with NBD C_6-ceramide in glucose-containing media (SD) showed that they synthesize only labelled IPC. In contrast the same mutant line, labelled under the same conditions, but complemented with TbSLS4 or TbSLS1 synthesized 2 predominant sphingolipid species, one of which co-migrated with IPC, the other with SM. In addition, in TbSLS4 complemented yeast a minor labelled species (X) was evident which co-migrated with an unknown detected in labelled mammalian cell (Vero) extracts, in which the predominant complex sphingolipid is SM.
B. The yeast IPC synthase (AUR1) is down-regulated in YPH499-HIS-GAL-AUR1 cells transformed with empty vector (pRS426) when incubated in non-permissive, glucose-containing media (SD). This is demonstrated by the lack of detectable IPC in yeast metabolically labelled with NBD C_6-ceramide in SD under the same conditions as above. In galactose-containing, glucose-free media (SGR) the synthesis of IPC is clearly evident.

NBD C_6-ceramide labelled lipid extracts fractionated by HPTLC, representative of at least 3 independent experiments. O, origin; IPC, inositol phosphorylceramide; SM, sphingomyelin; Cer, ceramide (migrating at the front); X, unknown sphingolipid. All lipid extracts normalised with respect to cell mass.

Figure 3

A. Sphingomyelinase (SMase) treatment of lipids extracted from YPH499-HIS-GAL-AUR1 complemented with TbSLS4, grown in glucose-containing media (SD) and labelled with NBD C_6-ceramide, demonstrated that the predicted sphingomyelin species is SMase sensitive, as is unknown X. In contrast the predicted IPC is insensitive. As a control, YPH499-HIS-GAL-AUR1 expressing human SM synthase 2 (HsSMS2, grown in permissive SGR media) was utilized as they produce equivalent quantities of labelled SM to the TbSLS4 line.
B. The yeast IPC synthase (AUR1) is down-regulated in YPH499-HIS-GAL-AUR1 cells transformed with empty vector (pRS426) incubated in non-permissive SD under the same conditions as the SMase treated samples. This is demonstrated by the lack of detectable IPC in yeast metabolically labelled with NBD C_6-ceramide in SD. In galactose-containing, glucose-free media (SGR) the synthesis of IPC is clearly evident.

NBD C_6-ceramide labelled lipid extracts fractionated by HPTLC, representative of at least 3 independent experiments. O, origin; IPC, inositol phosphorylceramide; SM, sphingomyelin; Cer, ceramide (migrating at the front); X, unknown sphingolipid. All lipid extracts normalised with respect to cell mass.

Figure 4

Detergent-washed microsome extracts from YPH499-HIS-GAL-AUR1 TbSLS4 yeast demonstrate IPC synthase enzyme turnover on the addition of the donor substrate PI and acceptor substrate NBD C_6-ceramide, but no further production of other sphingolipid species on the addition of alternative donors PC and PE.

A. The addition of bovine liver PI led to a marginal increase in IPC production in unwashed (Crude) AUR1 microsomes when compared to the control (-). In CHAPS-treated microsomes (Washed) IPC synthase (AUR1) turnover was minimal without donor substrate (-) and unchanged by the addition of either PI or PC as donors. This demonstrated that the yeast IPC synthase (AUR1) was unable to utilise these donor substrates under the experimental conditions.
B. In contrast, assay of TbLS4 microsomes (CHAPS-treated, as the Washed sample in A) demonstrated a greater than 12-fold increase in IPC over a sample without donor substrate (-) on the addition of PI. PC and PE had no demonstrable effect on enzyme turnover. However, both inhibited IPC synthesis when added together with PI at equivalent molar quantities: PC by approximately 4-fold and PE by approximately 2-fold.

NBD C_6-ceramide labelled lipid extracts fractionated by HPTLC and quantified as described, standard deviation of 3 independent experiments shown.

AFU, arbitrary fluorescence units; IPC, inositol phosphorylceramide; SM, sphingomyelin; -, no donor substrate added; PI, bovine liver phosphatidylinositol; PC, bovine liver phosphatidylcholine; PE, bovine liver phosphatidylethanolamine.

Figure 5

The fungal IPC synthase inhibitor aureobasidin A is active against TbSLS activity.

A. TbSLS4 IPC synthase turnover determined using the described *in vitro* assay in the presence of aureobasidin A (AbA). The IC_{50} was calculated from the linear portion of the curve as being 0.42 nM.
B. *Leishmania major* IPC synthase turnover was confirmed to be relatively refractory to AbA [10]. The IC_{50} was in excess of 100 μM, more than 200 000 times greater than that for TbSLS4.

Turnover, as determined by IPC production, was scored as 100% in the absence of the inhibitor.

Figure 6

TbSLS is an essential enzyme in bloodstream form *T. brucei*.

A. Inhibition RNA of TbSLS. Cell counts over a 48 hour period: ▲ Mock transfected cells non-induced; ✕ Mock tranfected cells induced by 1 μg/ml doxycline; ♦ TbSLS RNAi cells non-induced; ■ TbSLS RNAi cells induced by 1 μg/ml doxycline. Error bars for standard deviation over three replicates are shown.
B. RT PCR using total RNA isolated from TbSLS RNAi parasites with or without doxycycline induction (Doxy). RT, reverse transcriptase. βTUB, β tubulin control.

Figure 7

Aureobasidin A is trypanocidal against bloodstream form *T. brucei*. Cell counts over 72 hours with: ▲ 1 μM aureobasidin A (AbA); ■ 250 nM AbA; ♦ control. Error bars for standard deviation over three replicates are shown.

Figure 1

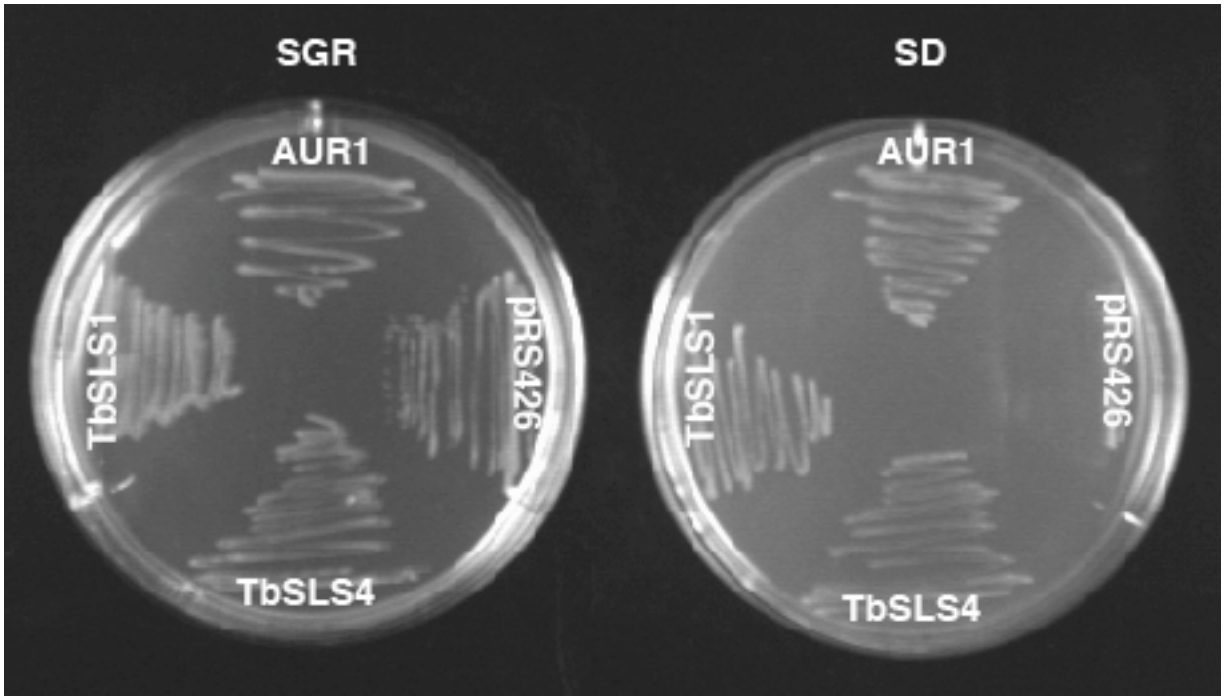

Figure 2

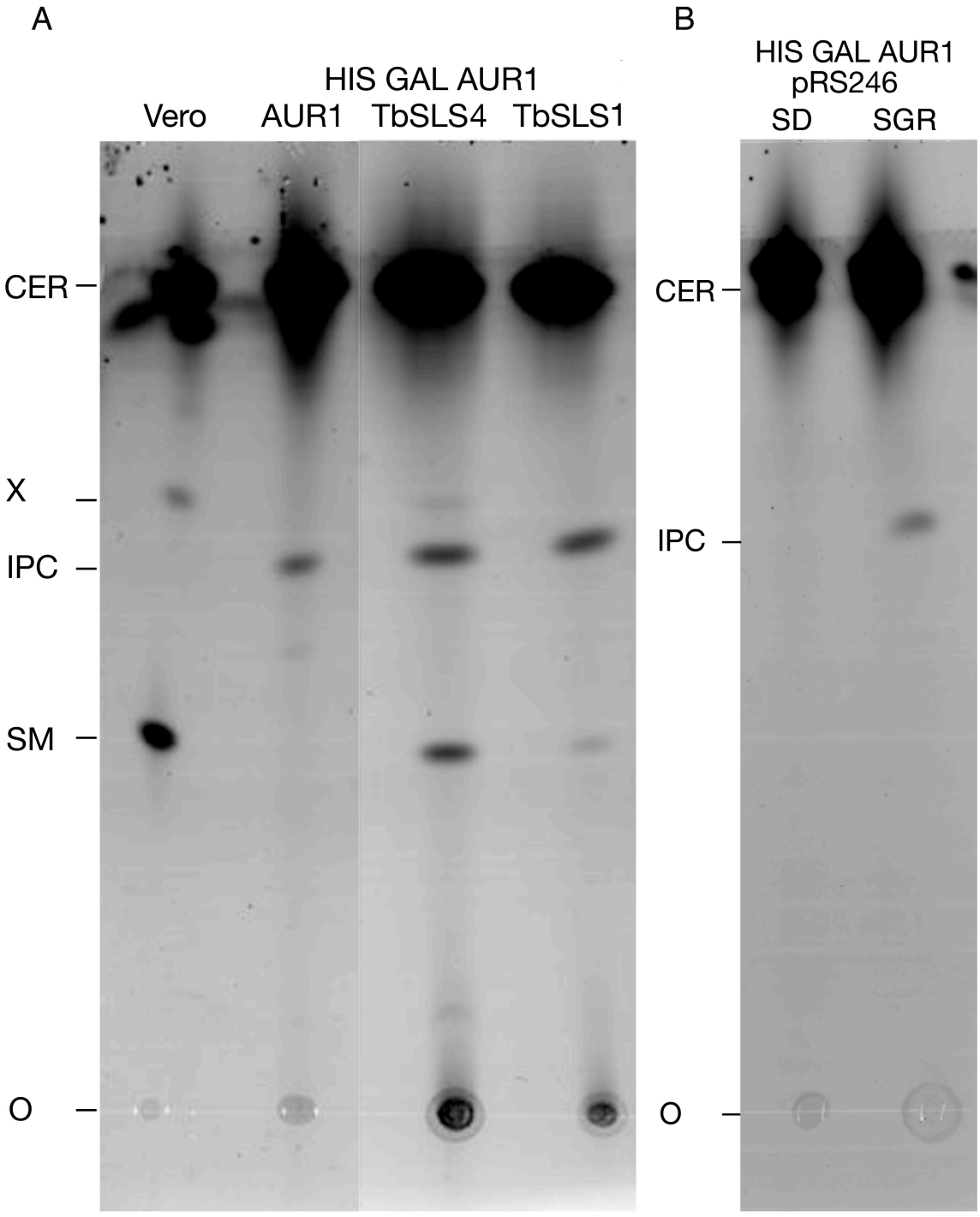
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Figure 3

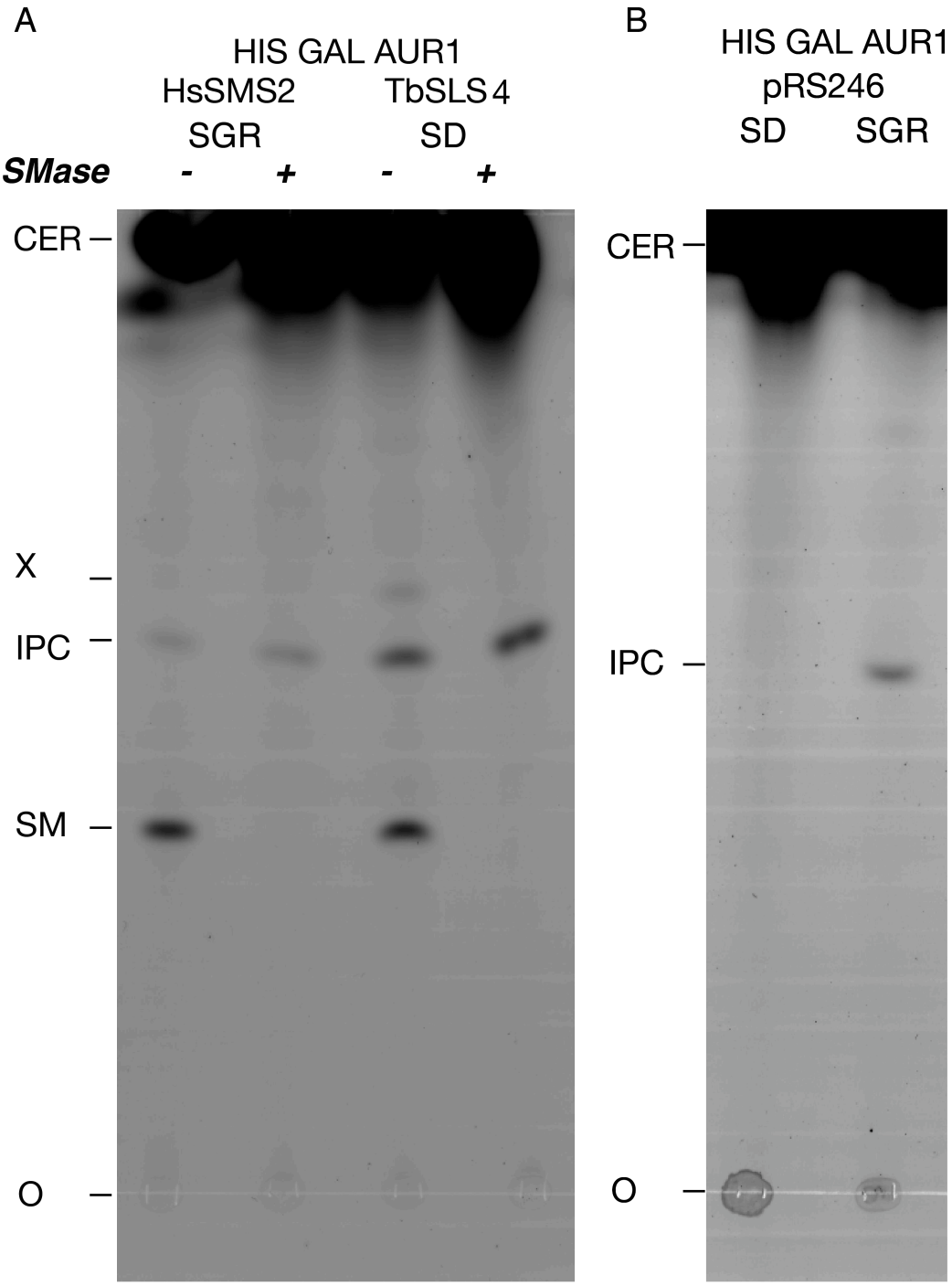
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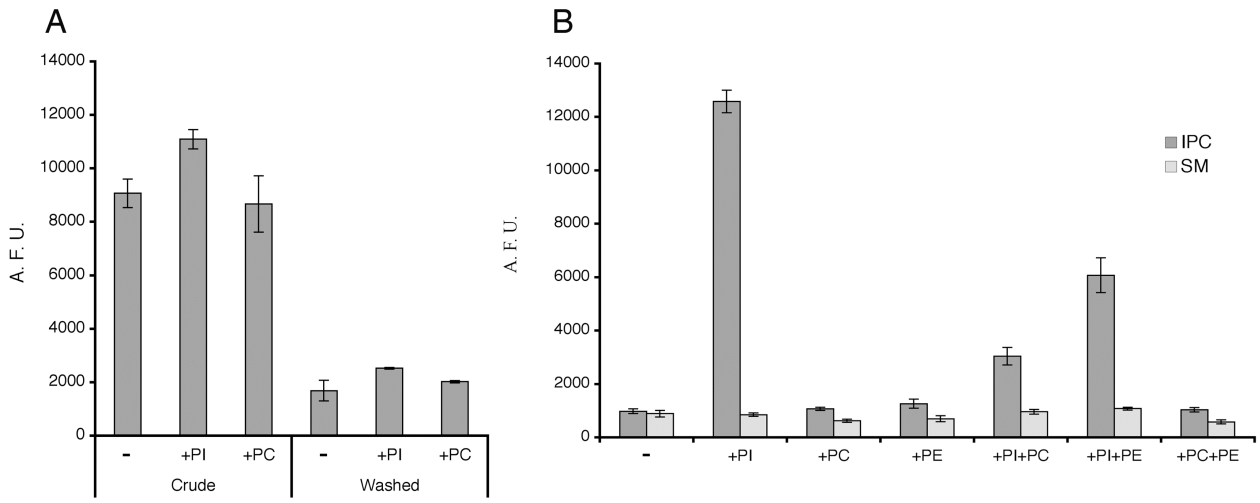
Figure 4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SM

Figure 5

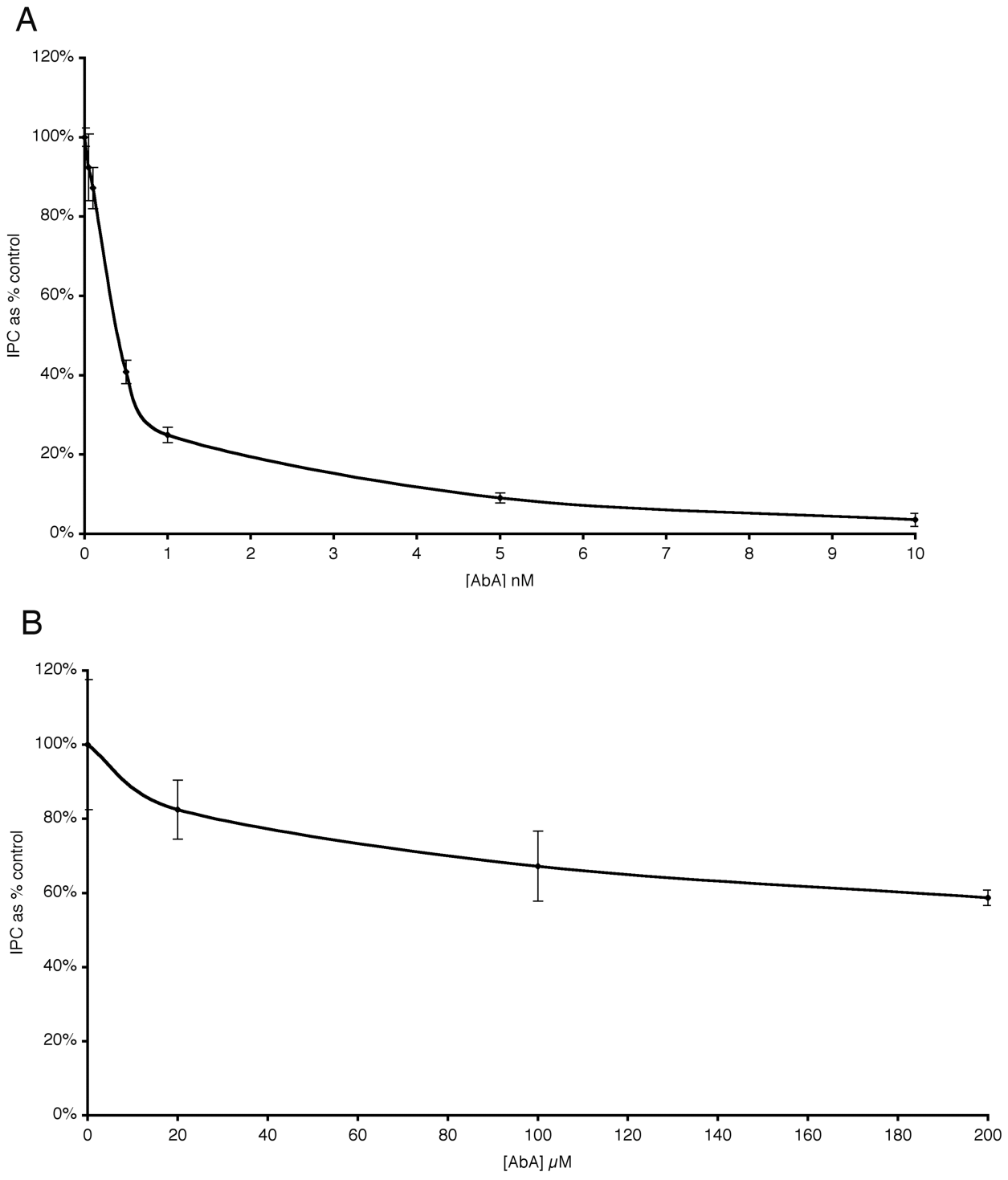
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Figure 6

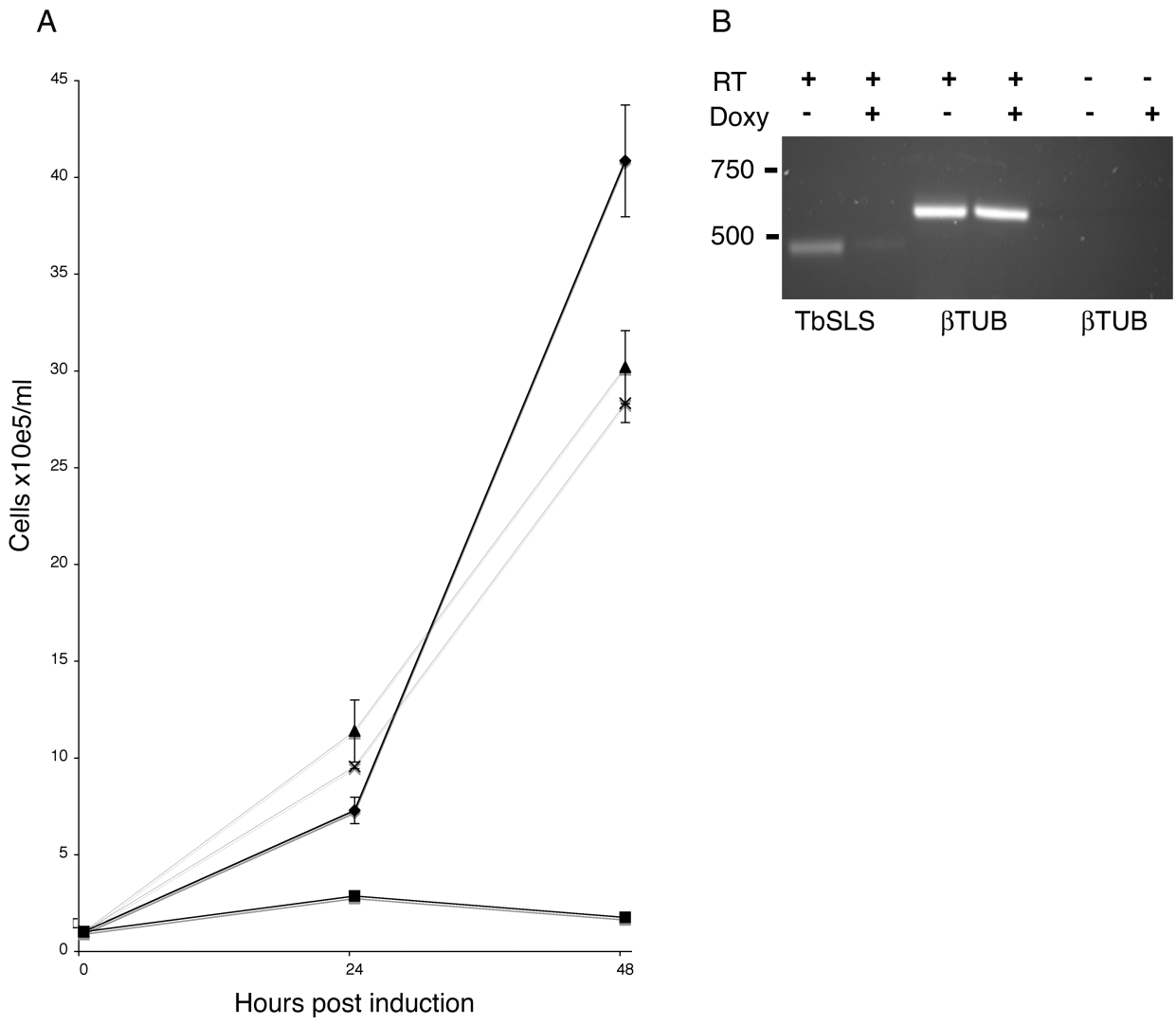
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Figure 7